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Impaired mitochondrial microbicidal responses in chronic obstructive pulmonary disease macrophages.

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Author contributions: JAP and MAB contributed equally to this work. JAP made and validated the transgenic mouse. MAB performed killing assays, flow cytometry and microscopy collected data and produced figures. MM performed Seahorse experiments, and imaging, HMM performed in vivo experiments and JS contributed to design of imaging experiments. RCB and DS co-ordinated and performed bronchoscopies to obtain patient samples. DRG designed the CD68 construct. RWC designed the Mcl-1 construct. LED designed experiments measuring COPD associated phagocytic defects. PJB and CEB co-ordinated collection of the COPD patient cohort. MKBW, SDS and DHD designed and conceived the experiments. JAP, MAB and DHD wrote the manuscript with input from all other authors.

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At a glance summary:

Scientific Knowledge on the Subject: COPD patients are at increased risk from bacterial respiratory infections, which cause acute exacerbations adding to morbidity. Previous studies have identified potential defects in innate immunity but the effect of COPD on macrophage microbicidal responses has been little investigated. Host-mediated macrophage apoptosis in response to bacteria gives an increment to bacterial killing once canonical phagolysosomal killing has become exhausted. Defects in this pathway alter bacterial clearance.

1 **What This Study Adds to the Field:** We show that inhibition of macrophage apoptosis
2 and a failure to induce mitochondrial reactive oxygen species generation in COPD
3 macrophages contributes to impaired clearance of pneumococci in the lung.

4 **Footnote:**

5 Presented in part as “Alveolar macrophages during COPD demonstrate reduced
6 apoptosis-associated bacterial killing and reduced mitochondrial reactive oxygen
7 species-dependent killing”. M. Bewley, R. Budd, D. Singh, P.J. Barnes, L.E. Donnelly,
8 M.K.B. Whyte, D.H. Dockrell, Medical Research Council COPD MAP Consortium.
9 American Thoracic Society International Conference San Diego May 19th, 2014. Am J.
10 Respir Crit Care Med 18:2014; A2862

1 **Abstract:**

2

3 **Rationale:** Chronic obstructive pulmonary disease (COPD) is characterized by impaired
4 clearance of pulmonary bacteria.

5 **Objectives:** The effect of COPD on alveolar macrophage (AM) microbicidal responses
6 was investigated.

7 **Methods:** Alveolar macrophages (AMs) were obtained from bronchoalveolar lavage
8 from healthy donors or COPD patients and challenged with opsonized serotype 14
9 *Streptococcus pneumoniae*. Cells were assessed for apoptosis, bactericidal activity and
10 mitochondrial reactive oxygen species (mROS) production. A transgenic mouse line, in
11 which the CD68 promoter ensures macrophage specific expression of human Mcl-1
12 (CD68.hMcl-1), was used to model the molecular aspects of COPD.

13 **Measurements and Main Results:** COPD AM had elevated levels of Mcl-1, an anti-
14 apoptotic Bcl-2 family member, with selective reduction of delayed intracellular
15 bacterial killing. CD68.hMcl-1 AM phenocopied the microbicidal defect since
16 transgenic mice demonstrated impaired clearance of pulmonary bacteria and increased
17 neutrophilic inflammation. Murine bone marrow-derived macrophages (BMDM) and
18 human monocyte-derived macrophages (MDM) generated mitochondrial reactive
19 oxygen species (mROS) in response to pneumococci, which co-localized with bacteria
20 and phagolysosomes to enhance bacterial killing. The Mcl-1 transgene increased oxygen
21 consumption rates and mROS expression in mock-infected BMDM but reduced caspase-
22 dependent mROS production after pneumococcal challenge. COPD AM also increased
23 basal mROS expression, but failed to increase production after pneumococcal challenge,
24 in keeping with reduced intracellular bacterial killing. The defect in COPD AM
25 intracellular killing was associated with a reduced ratio of mROS /superoxide dismutase
26 2.

1 **Conclusions:** Upregulation of Mcl-1 and chronic adaption to oxidative stress alters
2 mitochondrial metabolism and microbicidal function, reducing the delayed phase of
3 intracellular bacterial clearance in COPD.

4

5 Word count: 250

6 Key words: Apoptosis, mitochondrial reactive oxygen species, Streptococcus
7 pneumoniae.

1 **Introduction**

2 Chronic obstructive pulmonary disease (COPD) is characterized by incompletely
3 reversible airway obstruction. Neutrophilic inflammation drives airway narrowing and
4 alveolar destruction (1). Cigarette smoke and biomass fuels are major factors initiating
5 COPD pathogenesis and persistent neutrophilic inflammation in those who quit smoking
6 emphasizes the importance of additional etiologic factors in maintaining progressive
7 airway destruction (2, 3).

8

9 Clinical exacerbations of COPD, punctuate periods of relative stability in many patients,
10 and contribute to the decline in respiratory function (4). Exacerbations are frequently
11 associated with the presence of pathogenic bacteria in the lower airway and the lower
12 airway bacterial load correlates with markers of neutrophilic inflammation such as
13 CXCL8 levels (5). This suggests that a key driver of COPD pathogenesis is a defect in
14 airway innate immune responses to pathogenic bacteria. While *Haemophilus influenzae*,
15 *Streptococcus pneumoniae* and *Moraxella catarrhalis* are all associated with infective
16 exacerbations, *S. pneumoniae* remains the major cause of community-acquired
17 pneumonia (CAP) in COPD (6, 7). The relative risk of CAP, pneumococcal CAP and
18 invasive pneumococcal disease (IPD) is elevated in patients with COPD to a greater
19 extent than smokers another group at increased risk of IPD (8). This suggests that
20 patients with COPD possess significant defects in their host defences to pneumococcal
21 disease in the lower airway. However the basis of this increased susceptibility to
22 pneumococcal pneumonia remains undefined.

23

24 Alveolar macrophages (AM) are central to the organization of pulmonary innate
25 immunity and are critical for clearance of pneumococci from the alveolar space (9).
26 However, AM do not possess several of the microbicidal molecules used by neutrophils

1 and pathogens have acquired adaptations to resist others (10), which challenges AM
2 microbicidal capacity. Macrophages therefore employ additional host defence strategies
3 and induction of apoptosis is required for efficient clearance of intracellular bacteria
4 after phagocytosis (9, 11). Apoptosis is controlled by expression of the anti-apoptotic
5 protein Mcl-1, which is dynamically regulated after bacteria are internalized (12, 13).
6 COPD is associated with decreased macrophage innate competence as illustrated by
7 evidence for impaired bacterial phagocytosis of non-typeable *H. influenzae* and *S.*
8 *pneumoniae*, the pathogens that most frequently colonize the lower airway in COPD (14,
9 15). Little is known, however, concerning the effect of COPD on AM microbicidal
10 responses.

11
12 We observed that COPD AM have persistent upregulation of Mcl-1 and have used
13 patient AM and a unique murine transgenic macrophage to test how Mcl-1 upregulation
14 alters pulmonary antibacterial host defense. Specifically, we addressed how Mcl-1
15 influenced macrophages ability to generate a mitochondrial microbicidal response
16 involving generation of mitochondrial reactive oxygen species (mROS) in response to *S.*
17 *pneumoniae* and how this influenced intracellular bacterial killing. Some of the results
18 of these studies have been previously reported in the form of an abstract (16).

19 **Materials and Methods.**

20 **Bacteria and Infection**

21 Serotype 2 *S. pneumoniae* (D39 strain, NCTC 7466) , serotype 1 *S. pneumoniae* (WHO
22 reference laboratory strain SSISP 1/1: Statens Seruminstitut), used in murine
23 experiments (9) and serotype 14 *S. pneumoniae* (NCTC11902) used in COPD
24

1 experiments were cultured and opsonized in human (11) or murine serum before
2 infection of cells as previously described (13).

3 **Isolation and culture of macrophages**

4 Bone marrow-derived macrophages (BMDM) were obtained as described (9). Human
5 monocyte-derived macrophages (MDM) were isolated from whole blood donated by
6 healthy volunteers with written informed consent, as approved by the South Sheffield
7 Regional Ethics Committee (11). AM from patients with COPD (enrolled through MRC
8 COPD-MAP) or from healthy controls (online data supplement Table E1) were isolated
9 from BAL as previously described (16), with written approved consent prior to inclusion
10 in the study as approved by the National Research Ethics Service Committee for
11 Yorkshire and the Humber. Further information can be found in the online supplement.

12

13 **Western blot**

14 Whole cell extracts were isolated using SDS-lysis buffer and separated by SDS gel
15 electrophoresis. Detailed information can be found in the online supplement.

16

17 **Immunohistochemistry**

18 Preparation of healthy and COPD lung sections (online data supplement Table E2),
19 immunostaining and semi-quantitative evaluation, are described in the online
20 supplement.

21

22 **Flow Cytometry**

23 Caspase activity was measured using the CellEvent caspase 3/7 green flow kit (Life
24 Technologies), according to the manufacturers instructions. Mitochondrial reactive

oxygen species (mROS) were measured by flow cytometry using the dye MitoSOX-Red (Invitrogen). Detailed information can be found in the online supplement.

Microscopy

Nuclear fragmentation and condensation indicative of apoptosis were detected using 4'6'-diamidino-2-phenylindole (DAPI) (11). To visualise mROS cells were stained with 2µM MitoSOX-red (Invitrogen) and visualized on a Leica DMRB 1000, 40x objective. For co-localisation experiments, lysosomes were stained with 0.50µM Cresyl violet (Sigma) or challenged with D39 labelled with Alexa Fluor 647 carboxylic acid succinimidyl ester (Life Technologies) and co-stained with MitoSOX-red for 15 min, and visualised by confocal microscopy (Zeiss LSM 510, 63x1.4 oil objective). In other experiments, MDM and BMDM were challenged with D39 labelled with Alexa Fluor 647 and 16 h post-challenge cells were stained with MitoSOX and visualised by structured illumination microscopy (SIM). Detailed information can be found in the online supplement.

Intracellular killing assay.

Assessment of intracellular bacterial viability was carried out by gentamicin protection assay as previously described (17).

Metabolic measurements

Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were performed using the XF24 extracellular flux analyser (Seahorse, Bioscience). Additional information can be found in the online supplement.

1 **In vivo infections**

2 Mcl-1 transgenic mice and wild-type littermates were infected and analyzed as outlined
3 in the online supplement. Animal experiments were conducted in accordance with the
4 Home Office Animals (Scientific Procedures) Act of 1986, authorized under UK Home
5 Office License 40/3251 with approval of the Sheffield Ethical Review Committee,
6 Sheffield, United Kingdom.

7

8 **Statistics**

9 Data are represented as mean and standard error unless otherwise indicated in the figure
10 legends. Sample sizes were informed by standard errors obtained from similar assays in
11 prior publications (12, 13). Analysis was performed with tests, as outlined in the figure
12 legends, using Prism 6.0 software (GraphPad Inc.) and significance defined as $p < 0.05$.
13 Decisions on the use of parametric (for normally distributed data) or non-parametric
14 tests (for non-normally distributed data) were informed by the distribution of data.

15

16 **Results**

17 Mcl-1 is upregulated and is associated with reduced intracellular bacterial killing in
18 COPD AM.

19 The Bcl-2 family member Mcl-1 regulates both macrophage viability (18) and delayed
20 bacterial killing through induction of apoptosis during exposure to bacteria such as the
21 pneumococcus (12, 13). We therefore investigated whether Mcl-1 expression was
22 altered in AM by COPD. These experiments were conducted with a strain of *S.*
23 pneumoniae that frequently colonizes this patient group and which also can cause IPD
24 (19). Since macrophages from patients with COPD have an impaired capacity to ingest
25 bacteria (14, 15), we modified the inoculum presented to control human AM to

1 normalize intracellular bacterial numbers (supplemental Figure E1) and demonstrated
2 significant reduction in Mcl-1 expression in healthy donor's AM but no reduction in
3 expression in COPD AM after bacterial challenge (Figure 1A). We also examined
4 whether there was increased Mcl-1 expression in AM in the COPD lung. As illustrated
5 quantification revealed enhanced Mcl-1 expression in lung biopsies of patients with
6 COPD compared to control donors without COPD, which showed a focal distribution of
7 high intensity (Figure 1B-D). The level of expression in controls was not altered by
8 whether these were current smokers or non-smokers and results were similar when
9 expression was analysed either by individual cell expression (Figure 1C) or by donor
10 overall (Figure 1D).

11

12 We next addressed whether COPD AM had any defects in early microbicidal responses.
13 In keeping with prior reports with MDM (14), we documented reduced phagocytosis of
14 pneumococci by COPD AM (Figure 2A). Of note there was no evidence of the normal
15 opsonic uplift in phagocytosis of pneumococci in COPD AM (16) in contrast to those
16 from healthy donors. Despite differences in initial internalization of opsonized bacteria
17 and COPD there was no evidence of significant reduction in early intracellular bacterial
18 killing, which is linked to initial bacterial phagocytosis in myeloid cells and reactive
19 oxygen species (ROS) generation via nicotinamide adenine dinucleotide phosphate-
20 oxidase (20). To exclude confounding effects of differential bacterial internalization we
21 adjusted the MOI used to challenge control AM, ensuring normalization of initial
22 internalization (supplemental Figure E1), since apoptosis is directly related to initial
23 bacterial ingestion (11). Upregulation of Mcl-1 was associated with reduced apoptosis in
24 COPD AM (Figure 2B) and with enhanced survival of intracellular bacteria at a later
25 time point (Figure 2C), supporting a defect in delayed apoptosis-associated
26 pneumococcal killing by AM (12, 13).

1

2 Mcl-1 upregulation in AM impairs bacterial clearance in the lung.

3 CD68.hMcl-1⁺ transgenic mice were used to explore the functional consequence of Mcl-

4 1 upregulation for bacterial clearance and the putative association of Mcl-1 upregulation

5 with altered intracellular bacterial clearance in COPD AM. Expression of a human Mcl-

6 1 transgene in myeloid cell populations extends macrophage survival while ensuring

7 cells remain sensitive to physiological constraints on viability so that there is normal

8 distribution of myeloid subsets and development (21). Using a low dose of

9 pneumococci, which AM are able to contain (9), we demonstrate that the presence of the

10 macrophage transgene results in impaired bacterial clearance from the lung and also

11 increased bacteremia (Figure 3A-B). These changes were found in association with

12 reduced AM apoptosis (Figure 3C) and increased numbers of neutrophils in the

13 bronchoalveolar lavage fluid (BAL) (Figure 3D).

14

15 Mcl-1 modulates generation of mitochondrial ROS and mROS-dependent bacterial

16 killing.

17 We next explored the links between induction of the Mcl-1 regulated apoptotic program

18 and microbicidal responses, using BMDM as a model of differentiated macrophages.

19 Mcl-1 regulates apoptosis at the level of the mitochondrion (12, 13) and mitochondrial

20 ROS (mROS) has emerged as an important microbicidal strategy used by macrophages

21 (22). mROS was significantly increased in CD68.hMcl-1⁻ BMDM (but not CD68.hMcl-

22 1⁺) 20h after bacterial challenge, with significantly lower levels in CD68.hMcl-1⁺

23 compared to CD68.hMcl-1⁻ BMDM (Figure 4A-B). mROS co-localized with

24 phagolysosomes and with bacteria, in contrast to endoplasmic reticulum, used as a

25 control, which did not co-localize with either bacteria or phagolysosomes (Figure 4C-F

26 and supplemental Figure E2-3). mROS staining was inhibited by an inhibitor

1 mitoTEMPO and each stain provided minimal background signal (supplemental Figure
2 E2-4). mROS co-localization with bacteria was also visible in human MDM (Figure 4E
3 and supplemental Figure E2F-G). mitoTEMPO, blocked the delayed phase of
4 pneumococcal killing in CD68.hMcl-1⁻ (but not CD68.hMcl-1⁺) BMDM (Figure 4G)
5 and also in MDM (Figure 4H).

6
7 Mcl-1 modulates mitochondrial oxidative phosphorylation in macrophages.

8 Generation of mROS occurs during oxidative phosphorylation when electron leak
9 predominantly from complex I results in generation of superoxide (23). We examined
10 whether Mcl-1 modulates oxidative metabolism. As anticipated pneumococcal infection
11 enhanced glycolytic metabolism (Figure 5A), in keeping with the known enhancement
12 of glycolytic metabolism during macrophage responses to bacteria (24), but the
13 CD68.hMcl-1 transgene did not alter glycolytic metabolism after infection. Also as
14 expected infection was associated with a reduction in several parameters associated with
15 oxidative phosphorylation, but the transgene itself resulted in increased baseline and
16 maximal oxygen consumption rate (OCR) in mock infected cells, though it had no effect
17 on the levels after pneumococcal challenge (Figure 5B-F). In association with alterations
18 in OCR parameters the transgene was also associated with increased baseline mROS
19 levels in mock-infected cells (Figure 5G). Since caspase activation enhances mROS
20 production through interference with complex I of the electron transport chain (25), we
21 next tested if Mcl-1 inhibited the inducible mROS expression observed after
22 pneumococcal challenge in a caspase-dependent process and whether this overwhelmed
23 Mcl-1's baseline effects on oxidative phosphorylation. As shown in Figure 5H-I, we
24 show that Mcl-1 overexpression inhibits the increase in mROS production following
25 bacterial challenge. Mcl-1 also specifically reduced the mROS production due to
26 caspase activation after bacterial challenge, as treatment with the casapase inhibitor

1 zVAD reduced MitoSOX levels to comparable levels to those of the Mcl-1 transgenic.
2 In these experiments with zVAD and zFA the baseline level of mROS was lower than in
3 the experiments in Figure 5G, reflecting reduced sensitivity of detection in the presence
4 of these chemicals, and the baseline alteration in mROS production due to the transgene
5 was no longer apparent.

6 7 COPD AM fail to increase mROS production after pneumococcal challenge

8 Since COPD patients had enhanced expression of Mcl-1 in AM (Figure 1 A-C),
9 phenocopying the CD68.hMcl-1⁺ BMDM we next addressed whether they also had
10 modulation of mROS generation in response to pneumococci and whether this
11 influenced intracellular bacterial killing. AM from patients with COPD had enhanced
12 levels of mROS at baseline and no increment with infection (Figure 6A). Inhibition of
13 mROS in COPD AM did not increase intracellular bacterial numbers, suggesting mROS
14 plays little role in bacterial killing in COPD AM (Figure 6B). COPD is associated with
15 enhanced antioxidant expression as an adaptation to chronic production of ROS (26).
16 The major antioxidant against superoxide in AM that is localized to mitochondria is
17 manganese superoxide dismutase (MnSOD/SOD2) (27). AM from COPD patients had
18 enhanced expression of SOD2 at baseline and maintained expression following bacterial
19 challenge (Figure 6C). Thus, when we calculated a ratio of the change in mROS to
20 SOD2 as a marker of mitochondrial oxidant/antioxidant balance, there was a significant
21 increase in this ratio following infection in healthy but not COPD AM (Figure 6D). To
22 test whether increased mROS reconstituted bacterial killing in COPD AM, we added the
23 mitochondrial complex I inhibitor rotenone, which enhances mROS production (28),
24 confirming it enhanced bacterial killing in both healthy and COPD AM (Figure 6E). In
25 keeping with a limited role for mROS in induction of apoptosis under these
26 circumstances, and the well developed resistance of AM to oxidative stress (29), we

1 found that rotenone resulted in only a limited increase in AM apoptosis. Once again this
2 suggested mROS was an effector of bacterial killing downstream of apoptosis rather
3 than a stimulus for apoptosis induction (Figure 6F).

6 **Discussion**

7 We demonstrate that COPD AM possess a specific defect in the delayed phase of
8 intracellular bacterial killing in association with impairment of mROS generation. This
9 phase of bacterial killing is regulated by the anti-apoptotic protein Mcl-1 (12, 13) and
10 we provide evidence that Mcl-1 is upregulated in COPD AM. Using a novel transgenic
11 mouse line in which human Mcl-1 is governed by the CD68 promoter we show that
12 overexpression of Mcl-1 results in a reduction of bacterial clearance from the murine
13 lung and that mROS is both required for the delayed phase of clearance and regulated
14 via Mcl-1 expression. Mcl-1 enhances the oxygen consumption rate during oxidative
15 phosphorylation and mROS production in mock-infected macrophages but during
16 infection its major effect is to regulate caspase dependent mROS production. COPD AM
17 have both high basal mROS generation and a failure to enhance mROS production after
18 pneumococcal challenge, which results in decreased bacterial killing.

20 COPD is characterized by bacterial persistence in the airway and by enhanced rates of
21 CAP and IPD (8, 30). Bacterial load in the airway correlates with progressive airway
22 obstruction and maintenance of neutrophilic inflammation (5). Moreover, a recent
23 murine model, involving polymeric immunoglobulin deficient mice, has demonstrated
24 that persistent exposure to lung bacteria drive inflammatory changes and lung
25 remodelling in the small airways (31). This suggests that innate immune dysfunction and

1 impaired handling of respiratory pathogens is a central feature of COPD pathogenesis.
2 In line with these observations several groups have demonstrated that COPD AM have
3 altered activation states (32, 33) cytokine responses (34) and phagocytic capacity (15,
4 35, 36). Despite this there has been little investigation of microbicidal responses in
5 COPD AM. Moreover prior studies have varied in the extent to which they identify a
6 systemic versus a local alveolar macrophage defect. Although confounding effects of
7 smoking and corticosteroids are important considerations we had low rates of current
8 smokers in the main patient group studied and although corticosteroid inhaler use was
9 more frequent in the COPD group the patients studied by histochemistry contained very
10 few who used corticosteroid inhalers, arguing against a major confounding effect of
11 these on Mcl-1 expression.

12

13 AM require additional microbicidal mechanisms to compliment early phagolysosomal
14 bacterial killing since they lack myeloperoxidase (37) and the granule-associated serine
15 proteases found in neutrophils (29). Moreover differentiated macrophages continue to
16 phagocytose bacteria after conventional phagolysosomal microbicidal strategies are
17 exhausted (38). Respiratory pathogens also express genes enabling their resistance to
18 microbicides (10). AM respond by activating a delayed phase of intracellular killing to
19 diverse pathogens ranging from pneumococci to *Mycobacterium tuberculosis* (12, 13,
20 39). Generation of mROS has emerged as an important microbicidal strategy used by
21 macrophages (22) and its production is increased by caspase 3 mediated inhibition of
22 complex I (25). Therefore it is well positioned to link induction of apoptosis to bacterial
23 killing. In light of observations that bacteria such as pneumococci have adaptations to
24 withstand oxidative stress, it is likely that mROS reacts to form other more potent
25 microbicides, such as reactive nitrogen species, to mediate bacterial killing (10). Our

1 results suggest that this critical microbicidal strategy functions ineffectively in COPD
2 AM.

3

4 Mitochondrial function emerges as a key determinant of the COPD AM microbicidal
5 response. In COPD there is increasing evidence of mitochondrial dysfunction involving
6 airway smooth muscle cells and skeletal muscle (40, 41). Enhanced mROS production is
7 well described and is believed to contribute to COPD pathogenesis by contributing to
8 the overall oxidative stress, promoting senescence and inflammation. The impact of
9 mitochondrial dysfunction on macrophage innate immune responses is less appreciated
10 but our data suggests that an additional consequence is impaired macrophage
11 microbicidal responses. These are likely to be compounded further by impaired
12 generation of classical macrophage activation in COPD (32, 33). Failure to generate
13 classical activation, will reduce succinate generation an important driver of acute mROS
14 production, required for microbicidal responses (42, 43), while the chronic production of
15 mROS can favour alternative activation with consequences to innate immune responses
16 (44). Upregulation of anti-oxidant defences, such as SOD2, in COPD (26), will further
17 compromise mitochondrial microbicidal capacity. Our results suggest AM adaptations to
18 chronic mROS generation will compromise the ability to generate an acute microbicidal
19 response with mROS in the phagolysosome.

20

21 The molecular regulation of mROS production involves Mcl-1. CD68.hMcl-1⁺ transgene
22 expression increased markers of oxidative phosphorylation and mROS generation in
23 mock-infected macrophages so its upregulation could theoretically drive chronic mROS
24 production in COPD AM. The production of mROS is, however, influenced by more
25 than the basal and maximal oxygen consumption rate and we do not know how COPD
26 influences hydrogen ion leak and the function of uncoupling proteins in AM in COPD

1 (23). Mcl-1 can exist as a form that localizes to the outer mitochondrial membrane and
2 regulates apoptosis and another form that localizes to the mitochondrial matrix and
3 enhances oxidative phosphorylation, while limiting mROS generation (45). This
4 suggests that if Mcl-1 is to contribute to enhanced mROS expression in COPD AM
5 either the expression of the matrix localized form must be altered or there are additional
6 factors modulating proton leak to result in greater mROS production. An additional
7 implication of this is that the reduced induction of mROS we document following
8 pneumococcal challenge in association with maintenance of Mcl-1 expression may not
9 just be a consequence of reduced caspase activation (and therefore inhibition of complex
10 I (25)) but may also result from preservation of the matrix localized form of Mcl-1 (45).
11 Regardless of these considerations there is still potential to overwhelm Mcl-1 in COPD
12 AM and re-engage both induction of mROS and microbicidal capacity, as evidenced by
13 the capacity of a complex I inhibitor to enhance bacterial clearance.

14

15 Our findings have been based exclusively on experiments with the pneumococcus but
16 are likely to have broad impact in COPD even though other bacteria such as non-
17 typeable *H. influenzae* are frequently implicated as colonizers of the COPD airway and
18 drive inflammation (46). Pneumococci are the second most frequently bacterial
19 colonizer in the lower airway in patients with COPD (46). They are likely to exert both
20 direct effects on the frequency of COPD exacerbations and decline in FEV1;
21 colonization with a monoculture of pneumococci specifically increased the risk of acute
22 exacerbation in one study (19), but also indirect effects since there is evidence that
23 pneumococcal colonization can promote *H. influenzae* or *M. catarrhalis* growth in the
24 upper airway or promote mixed *H. influenzae* and *S. pneumoniae* biofilms (47, 48).
25 They can also synergize with *H. influenzae* to promote pro-inflammatory cytokine
26 responses in epithelial cells (49). In addition they are the leading cause of CAP in

1 patients with COPD (7). However the induction of apoptosis-associated bacterial killing
2 is important against a range of pathogens (10), and the specific defect in mitochondrial
3 microbicidal responses is therefore likely to have consequences for other pathogens in
4 COPD beyond its effect on pneumococci.

5
6 Although several defects in innate immune function have been identified in COPD the
7 identification of a critical defect in the late phase of mitochondrial microbicidal killing
8 in COPD AM represents a new therapeutic target. Manipulation of mitochondrial
9 homeostasis, metabolism, or inhibition of Mcl-1, all represent potential approaches by
10 which this critical defect could be modified.

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1

2

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1

2 **Figure Legends**

3 **Figure 1: Mcl-1 upregulation occurs in chronic obstructive pulmonary disease**

4 **(COPD).** (A) Alveolar macrophages (AM) obtained from bronchoalveolar lavage of
5 healthy controls or COPD patients were mock-infected (MI) or challenged with
6 opsonized serotype 14 *Streptococcus pneumoniae* (S14) at the designated multiplicity
7 of infection (MOI). 16h post-challenge the levels of Mcl-1 on AM were probed by
8 western blot. A representative blot and densitometry are shown, n=6, *= p<0.05,
9 repeated measures 1-way ANOVA. **(B-C)** Lung sections from COPD patients or
10 healthy controls were dual stained with CD68 and Mcl-1. Total corrected cellular
11 fluorescence (TCCF) of Mcl-1 in CD68 +ve cells was quantified. Representative
12 images (B) and collated data (C and D) are shown. In C each point represents an
13 individual cell (n=74 healthy, n=90 COPD, from 10 donors) and in D, each point
14 represents the median fluorescence of all cells analysed from individual donors. For C
15 and D, *= p<0.05, Kruskal-Wallis test.

16

17 **Figure 2: COPD alveolar macrophages have a deficiency in apoptosis-associated**

18 **killing.** (A) Alveolar macrophages (AM) were collected from healthy donors or patients
19 with COPD and were challenged with non opsonized (-) or opsonized (+) serotype 14 *S.*
20 *pneumoniae* at an MOI of 10 for 4h, before extracellular bacteria were killed and viable
21 intracellular bacteria measured. Viable bacteria in duplicate wells were measured again
22 three hours later (7h post-infection), *= p<0.05. **= p<0.01, 2-way ANOVA. **(B-C)**
23 Healthy or COPD AM were challenged with S14, at an MOI of 10 for COPD cells, or
24 MOI 5 for healthy cells, so as to normalize levels of bacterial internalization. Cells were
25 analysed for (B) nuclear fragmentation or condensation and (C) intracellular bacterial

colony forming units (CFU) at 20h post-challenge n=5-6, *= p<0.05, Students t-test (for B) or Mann-Whitney U test (for C).

Figure 3: Mcl-1 upregulation in AM impairs bacterial clearance in the lung.

(A-D) Wild-type (Wt) or CD68.hMcl-1 transgenic (Tg) mice were challenged with 10⁴ serotype 1 *Streptococcus pneumoniae*. At the designated time after instillation, bacterial colony forming units (CFU) in the lung homogenate (A), CFU in the blood (B), alveolar macrophage (AM) nuclear fragmentation or condensation in bronchoalveolar lavage (BAL) (C) and total polymorphonuclear leukocyte (PMN) numbers in BAL (D) were measured. n=4-11 mice per group from three independent experiments, *= p<0.05 **= p<0.01, 2-way ANOVA.

Figure 4: Mcl-1 modulates generation of mitochondrial ROS and mROS-dependent

bacterial killing. (A) Wild-type (Wt) or CD68.hMcl-1 transgenic (Tg) bone marrow-derived (BMDM) were mock-infected (MI) or challenged with opsonized serotype 2 (D39) *S. pneumoniae*. 20 h post-challenge cells were stained with MitoSOX and visualised by microscopy to assess mitochondrial reactive oxygen species (mROS) generation. Images representative of three independent experiments, scale bar 50 µM.

(B) At the designated time post-challenge, mROS were also assessed by flow cytometry, n=3 *= p<0.05 D39 Wt vs. D39 Tg, 2-way ANOVA. (C) MI or D39 infected Wt BMDM were stained with Cresyl violet to detect lysosomes (green) and MitoSOX (red) at 20 h and analysed by confocal microscopy. Co-localized signals are yellow (Merge), scale bar 5 µm. (D) Confocal fluorescence microscopy of D39 BMDM challenged with Alexa Fluor 647 labelled bacteria (green) and stained with MitoSOX (red, 4D upper panels) or endoplasmic reticulum (ER) tracker (purple, 4D, lower panel) 20h after bacterial challenge. Co-localized signals are yellow (Merge, 4D upper and lower panels,

1 Scale bar 5 μ m). **(E)** Pseudo-coloured structured illumination microscopy (SIM) image
2 of a monocyte-derived macrophage 16 h after bacterial challenge with *S. pneumoniae*
3 (green) and stained with MitoSOX for mROS (red). Enlarged region on right shows
4 bacteria co-localized with mROS (arrows). Scale bar 10 μ m. **(F)** Pearson's correlation
5 coefficients were calculated for the co-localization of mROS or ER with D39 or
6 lysosomes, mean \pm SEM (n=4-8). **(G-H)** Wt or Tg BMDM (G) or human MDM (H)
7 were challenged with D39, in the presence or absence (vehicle) of mitoTEMPO (mT).
8 16 h post-challenge intracellular colony forming units (CFU) were assessed, n=5 (for G)
9 and n=8 (for H), **= $p < 0.01$, repeated measures 2-way ANOVA (for G), or Wilcoxon
10 matched-pairs signed rank test (for H).

11
12 **Figure 5: Mcl-1 modulates mitochondrial responses leading to mitochondrial**
13 **reactive oxygen species generation. (A-F)** Wild-type (Wt) or CD68.hMcl-1 transgenic
14 (Tg) bone marrow-derived (BMDM) were mock-infected (MI) or challenged with
15 opsonized serotype 2 (D39) *S. pneumoniae* for 4h before extracellular acidification
16 (ECAR) (A) and parameters related to oxidative phosphorylation were measured
17 kinetically. From the kinetic data (B), basal rates of oxygen consumption (OCR) (C),
18 maximum respiration capacity (D), ATP linked OCR (E), and proton leak (F) were
19 calculated, n=6 per group, *= $p < 0.05$ **= $p < 0.01$, 2-way ANOVA. **(G)** Mock-infected
20 Wt and Tg BMDM were stained with MitoSox to measure baseline mROS production.
21 **(H-I)** Wt or Tg BMDM (H) or human monocyte-derived macrophages (MDM) (I) were
22 MI or challenged with D39, in the presence of the pan-caspase inhibitor zVAD or
23 control zFA. At 20h post-challenge, cells were stained for mROS and caspase 3/7
24 activity. MitoSox staining was assessed for the whole cell populations (histograms).
25 Representative plots are shown, with collated data graphed, n=4 *= $p < 0.01$, 2-way
26 ANOVA (for H), or 1-way ANOVA (for I).

1

2 **Figure 6. COPD AM fail to increase mROS production after pneumococcal**

3 **challenge. (A-B)** Alveolar macrophages (AM) obtained from bronchoalveolar lavage

4 (BAL) of healthy controls (Healthy) or COPD patients (COPD) were mock-infected

5 (MI) or challenged with opsonized serotype 14 *S. pneumoniae* (S14), at an MOI of 10

6 for COPD cells, or MOI 5 for healthy cells. AM were left unstained (US) or stained

7 with MitoSOX and mean fluorescence intensity (MFI) recorded at 16h, as a measure of

8 mitochondrial reactive oxygen species (mROS), with representative plots shown and

9 collated data graphed, (A) and intracellular bacterial colony forming units (CFU) were

10 estimated in the presence or absence (vehicle) of mitoTEMPO (mT) (B) at 20h. Both

11 $n=6$, $^*=p<0.05$, paired Students t-test (A) or Wilcoxon signed rank test (B). (C) AM

12 from BAL of healthy controls or COPD patients were mock-infected (MI) or

13 challenged with S14 at the designated multiplicity of infection (MOI). At 16h post-

14 challenge the levels of superoxide dismutase (SOD)2 in AM were probed by western

15 blot. Representative blot and densitometry are shown, $n=4$. (D) The ratio of mROS to

16 SOD2 induced by bacterial challenge was calculated for healthy and COPD AM, using

17 the samples in (C), $n=4$, $^*=p<0.05$, Students t-test. (E-F) Healthy or COPD AM were

18 MI or challenged with S14, in the presence (+) or absence (-) of rotenone to induce

19 mROS. AM were assessed for intracellular bacterial CFU (E) and nuclear

20 fragmentation or condensation (F) 20h post-challenge, $n=3$, $^*=p<0.05$, Wilcoxon

21 signed rank test.

22